

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02030 A2

(51) International Patent Classification: A61L 27/00

(21) International Application Number: PCT/IL00/00386

(22) International Filing Date: 4 July 2000 (04.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/345,138 6 July 1999 (06.07.1999) US(71) Applicant (for all designated States except US): RAMOT
UNIVERSITY AUTHORITY FOR APPLIED RE-
SEARCH & INDUSTRIAL DEVELOPMENT LTD.
[IL/IL]; Haim Levanon Street 32, 69975 Ramat-Aviv (IL).

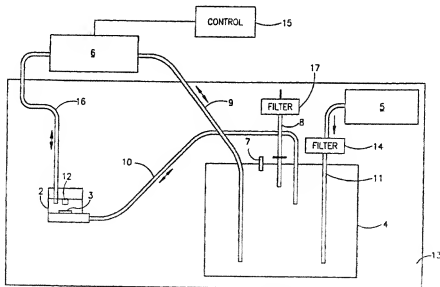
(72) Inventors; and

(75) Inventors/Applicants (for US only): NEVO, Zvi [IL/IL];
Yair Stern St. 11, 46425 Herzliya (IL). ROBINSON, Dror
[IL/IL]; Kfar Shmuel 100, 99788 D.N. Shimshon (IL).(74) Agent: REINHOLD COHN AND PARTNERS; P.O.
Box 4060, 61040 Tel-Aviv (IL).(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW). Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— Without international search report and to be republished
upon receipt of that report.For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: SCAFFOLD MATRIX AND TISSUE MAINTAINING SYSTEMS



(57) Abstract: The invention concerns a device with a constant perfusion system for maintenance of viable cells, tissues and composite implants, which involves a pressure generator for producing rhythmic pulses of pressure on the biological specimen. All the operative parameters of the system are controllable, easily manipulated. The invention further concerns a scaffold which is used as a growth supportive base for various cells and tissue explants from three-dimensional tissue comprising naturally derived connective or skeletal tissue cross-linked with one of the following: hyaluronic acid, proteoglycans, glycosaminoglycans, chondroitin sulfates, heparan sulfates, heparins and dextran sulfates.

SCAFFOLD MATRIX AND TISSUE MAINTAINING SYSTEMS

FIELD OF THE INVENTION

The invention concerns scaffold matrices for supporting three-dimensional tissues and systems for maintaining three-dimensional viable tissues.

BACKGROUND OF THE INVENTION

5 The following publications are believed to be relevant as background of the invention.

1. WO 98/22573
2. US 4,880,429
3. US 4,108,438
- 10 4. US 5,843,182
5. Mikos, A.G., Sarakinos, G., Leite, S.M., Vacanti, J.P., and Langer, R., "Laminated three-dimensional biodegradable foams for use in tissue engineering", *Biomaterials*, **14**:323-330, 1993.
6. Huttmacher, D., Kirsch, A., Ackeman, K.L., and Huerzeler, M.B., "Matrix and carrier materials for bone growth factors - state of the art and future perspectives in: Stark, G.B., Horch, T., Tancos, E. (eds). *Biological Matrices and Tissue Reconstruction*. Springer Verlag, 1998, pp. 197-203.
- 15 7. Kandel, R.A., Chen, H., Clark, J. and Renlund, R., *Transplantation of cartilaginous tissue generated in vitro into articular joint defects*. *Biotechnol.*, **23**:565-577, 1995.
- 20

Cartilage is a specialized form of connective tissue composed of cells and matrix. The cartilage cells synthesize matrix and become encased in cavities (lacunae) within it. The matrix is composed of fibers embedded in ground
25 substance and endows cartilage with its specialized physico-chemical properties.

Trauma, single or repetitive, and minute imbalance in joint stability are the most known causes of damage and degeneration of articular cartilage, that leads to pain, chronic disability and ultimately to joint failure. The current options for treatment provide temporary improvement of symptoms and function, however, there is no full restoration of joint performance. Prosthetic joint replacement is currently the ultimate and the most commonly employed treatment. Modern biological grafting is the other alternative for resurfacing the damaged joint, but is still imperfect.

A large number of candidate grafts have been studied for enhancing the repair of cartilage defects which include: (i) Osteochondral graft (autografts or allografts); (ii) Intact cartilage grafts; (iii) Growth plate; (iv) Isolated allogeneic chondrocytes; (v) Cultured autologous chondrocytes (dedifferentiated) (vi) Periosteum; (vii) Perichondrium; (viii) Bone marrow mesenchymal derived cells and (ix) Synovial membrane derived cells.

Another approach was the attempt to use natural occurring or synthetic biodegradable scaffolds which support three-dimensional growth of cartilage cells. The scaffolds may be impregnated with cells, which together with the scaffold form the graft. Alternatively, the scaffold may initially be devoid of impregnated cell, and endogenous cells from the patient are expected to migrate into the scaffold after its implantation.

Examples of such scaffolds are: (a) Fibrin polymers; (b) Collagene Type I; (c) Natural hyaluronic acid (HA) and chemically modified HA and (d) Synthetic bipolymers either biodegradable e.g. polylactic acid, polyglycolic acid or non-biodegradable (e.g. alginic acid). However, none of the above scaffolds can induce regeneration of hyaline-like cartilage. Fibrin adhesive polymers tend to induce dedifferentiation and thus do not permit production of functional tissue. Collagen Type I has no inherent chemotactic ability for chondrocytes, but stimulates proliferation of fibroblasts. Thus, instead of encouraging migration of chondrocytes, the tissue formed in this scaffold tends to be fibrous. Hyaluronic acid can stimulate chondrogenic differentiation, but does not stimulate chondrocytes

proliferation.. Alginic acid is a foreign sea weed derived carbohydrate and thus might induce an antigenic reaction, and furthermore it is not biodegradable. Polyglycolic and polylactic acid scaffolds do not support good hyaline cartilage regeneration due to acidic conditions formed during their degradation.

- 5 Damaged or missing hyaline cartilage is frequently repaired by transplantation of homografts. Homografts are immunologically privileged since the matrix acts as a barrier that permits only limited diffusion of high-molecular weight substances and contains an anti-angiogenesis factor to prevent invasion of host blood vessels and fibroblasts.
- 10 Various culturing systems have been developed for maintaining the viability and growth of tissues in culture. Generally, these are divided into static and perfusion bioreactors. Perfusion bioreactors are reactors which essentially keep constant, growth permissible conditions (such as nutrition, gas composition, temperature, pH, etc.) in which the growth fluid medium is constantly perfused in
- 15 and out of the system. Typically, perfusion is carried out by utilizing a constant velocity flow of the medium.

SUMMARY OF THE INVENTION

- By a first aspect, the present invention concerns a scaffold for use as growth
- 20 supportive base for cells and tissue explants from three-dimensional tissue, comprising a naturally derived connective or skeletal tissue which has been treated for elimination of cellular and cytosolic elements, and which has been modified by cross-linking with an agent selected from the group consisting of: hyaluronic acid, proteoglycans, glycosaminoglycan, chondroitin sulfates, heparan sulfates, heparins
- 25 and dextran sulfates.
- x-linking*

- It has been found that such a scaffold has the properties of encouraging cells' adherence thereto and enablement of propagation of cells on the one hand, while the cross-linking with the agents specified above gives the scaffold mechanical strength and produces a substance which is less brittle with prolonged
- 30 degradation time on the other hand. It was further found that the scaffold of the

invention supports chondrocyte proliferation at the expense of fibroblasts, resulting in a hyaline-like repair tissue.

The term "*scaffold*" in the context of the present invention refers to the connective/skeletal tissue which has been treated for elimination of cellular and
5 cytosolic elements and has been modified by cross-linking as described above, as well as to such a construct containing additional agents such as adhesive molecules or growth factors.

The term "*three-dimensional tissue*" (3D tissue) refers to any type of tissue which has an orderly three-dimensional structure, i.e., is not naturally present in the
10 body in the form limited to a single layer of cells or lamina, but has a structure which is spatially ordered. Examples of three-dimensional tissue are: mesenchymal tissue, cartilage and bone tissue, liver tissue, kidney tissue, neuronal tissue, fibrous tissue, dermis tissue etc. Another three-dimensional tissue is the whole embryonal epiphyseal organ derived from embryos at a post limb-bud stage.

15 The naturally derived connective or skeletal tissue is, in general, a tissue that was derived from mesenchymal tissues that expresses, temporarily or continuously, fibroblast growth factor receptor 3 (FGFR3). Examples of such tissue are mainly members of the chondrogenic and the osteogenic anlagen, as well as the residual mesenchymal stem cell reservoirs found in tissues all along life, ready to carry
20 wound healing, repair and regeneration tasks. Another example of connective or skeletal tissue is epiphyseal tissue, periosteal and perichondrial flaps that contain massive growth factors.

In order to turn a tissue into a scaffold, the tissue should be treated for elimination of cellular and cytosolic elements such as: DNA, RNA, proteins, lipids,
25 proteoglycans and in general most elements of the cells which are immunogenic, as well as treated for removal of calcification-mineralization centers. Methods for elimination of the above cellular and cytosolic elements are in general known in the art.

The naturally derived connective or skeletal tissue treated as described
30 above for elimination of cellular and cytosolic components is preferably further

treated for producing higher porosity by the production of pores in a controlled manner. The treatment may be mechanical, for example, by hammering the tissue on a scraper device, or by hammering a metal brush into the tissue (e.g. in epiphyseal tissue).

- 5 Alternatively, the treatment for producing porosity may be a chemical extraction process carried out by exposing the tissue, for a controlled amount of time, in a controlled environment to chemical agents capable of partial degradation of the tissue. In addition or alternatively, the treatment for producing porosity may be carried out by exposing the tissue to enzymatic agents such as proteolytic
10 enzymes, capable of partial degradation of the tissue. Example of such chemical agents which can produce pores in the tissue are guanidium chloride. The pores should have preferably a size of 10-500 μ , most preferably 20-100 μ .

The agents either specified in above (i.e. hyaluronic acid, proteoglycans, glycosaminoglycan, chondroitin sulfates, heparan sulfates, heparin and dextran
15 sulfates) or additional agents such as adhesive molecules or growth factor moieties may be linked to the residual scaffold either by sugar cross-linking, (for example using ribose and xylose), by carbodiimide or by 1, 1 carbonyl di-imidazole. Cross-linking with the above agents is generally carried out as known in the art of coupling in organic chemistry.

- 20 In accordance with the present invention, it is preferable that the scaffold also contains adhesive molecules in order to enhance cell adherence to the scaffold. Example of suitable adhesive molecules are the integrins and additional agents such as, laminin, fibronectin, hyaluronic acid, polylysine, lysozyme and collagen. The formation of collagen, for example, may be achieved by additions of ascorbic acid
25 and its stable derivative such as ascorbic-2-phosphate.

In accordance with the present invention, it is also preferable that the scaffold would contain endogenously or exogenously added growth factors, in order to enhance the rate of growth of the cells filling the three-dimensional space of the scaffold. Examples of suitable exogenously added growth factors are:
30 fibroblast growth factors (FGF's), TGF's, BMP's, IGF's. The growth factor chosen

should depend on the type of tissue used. It should be noted that scaffolds of natural tissues, devoid of cells and cytosolic elements may still contain endogenous growth factors, bound to extracellular matrix elements so that at times the endogenous growth factors present in the matrix are sufficient.

5 As indicated above, the adhesive molecules and growth factor molecules should be made part of the scaffold by cross-linking as explained above.

By one option, it is possible to formulate a prosthesis from the scaffold alone, i.e. of a scaffold devoid of cells. In such a case, the scaffold is formulated to a desired shape and is inserted into the desired location in the body of the
10 individual, for example, a location wherein it is desired to achieve invasion of endogenous mesenchymal cells such as in the knee joint,

The prosthesis is malleable and can be shaped as either a flat sheet of several millimeters in thickness or any other three-dimensional shape adapted to the shape of the lesion. Alternatively, the prosthesis, can, *a priori*, prior to implantation
15 contain embedded (impregnated) cells, for example cells grown originally as monolayers or multi-layers on filters (Millicell cell culture [PICMORG50] inserts for use in organotypic cultures, 30mm, low height, Millipore Corp. Bedford, MA, USA), and placed 5-10 units in the JSD, a device described further below, or tissue explants to allow their fast anchorage and integration into bone and cartilages.

20 The cells impregnating the prosthesis should preferably be from an autogeneic source, but can also be of an allogeneic source, as cartilage has a sort of an immunoprivilege.

The scaffold of the invention impregnated with cells may be used, not only for direct implantation in the body but also for prolonged *in vitro* growth and
25 differentiation of various three-dimensional tissues kinds such as skin, neuronal, bony, cartilaginous, liver, pancreatic beta cell and almost of any organ or tissue in a bioreactor, while adjusting the proper medium, cocktail of growth factors and adhesive molecules.

By another aspect the present invention concerns a system for maintaining
30 viable three-dimensional tissue.

Static cultures in regular incubators can support cell growth in monolayers, multilayers or at most few microns 50-100 micron of 3D explants. For larger 3D explants only special bioreactor devices can support growth by perfusing nutrients, gases and remove wastes.

- 5 In accordance with this second aspect, it was surprisingly found that for long-term maintenance of viable three-dimensional tissue, there is need to apply rhythmic pulses of pressure (hydrostatic, mechanical or shear force) in order to obtain optimal growth. For example for growing of an articular cartilage tissue there is an advantage in maintaining the tissue under reperative cycles of loads and
- 10 unloads of pressure in a rhythmic manner, simulating the natural growth conditions in the joint. The cellular mechanoreceptors seem to play a key role in this respect of cell growth.

The variables that can be manipulated in the system of the invention include stream flow velocity, amount (in atmospheres) of hydrostatic and/or mechanic

15 pressure, rhythmic action periods (frequency of applications of pressure) and pausal intervals (pulses), as well as change stream direction of the medium. By this second aspect, the present invention concerns a system for the maintenance of viable tissue comprising:

- (i) a chamber for holding the tissue, the chamber's atmosphere being
- 20 kept at a relatively constant gas composition, said gas composition being suitable for maintenance of viable biological tissues;
- (ii) a reservoir for holding tissue culture medium, said reservoir being in flow communication with the chamber;
- (iii) a pump for circulating the medium between the chamber and the
- 25 reservoir in a controlled manner; and
- (iv) a pressure generator for producing rhythmic pulses of pressure on the tissue present in the chamber.

The system of the invention is suitable for any type of cells or tissues, but is especially suitable for the growth of a three-dimensional tissue, according to the

30 definition above.

Basically, the system comprises a chamber for holding the tissue, the chamber's atmosphere being kept at relatively constant gas and temperature composition which are suitable for maintenance of viable biological tissue, for example 5% to 10% CO₂ in air at physiological temperature. This is usually
5 achieved by placing the chamber within a larger CO₂ incubator, capable of maintaining such an atmosphere, and ensuring that the atmosphere of the incubator in communication (as regards temperature and gas composition) with that of the chamber.

The system further comprises a reservoir for holding tissue culture medium
10 which is in flow communication with the chamber. Preferably, the size of the reservoir is about 30 to 100 times larger than that of the chamber for holding the tissue and is typically the size of 400-1000 ml. The medium in the reservoir of course contains the nutrients and various agents such as growth factors, etc. required for maintaining viability and growth of the tissue.

15 The system comprises a pump which circulates the growth medium between the chamber and the reservoir in a controlled manner. The pump may be a constant pump or a peristaltic pump utilizing either computerized or electrical/electronic manipulated regimes, as will be explained hereinbelow. Typically, the velocity of medium flow is in the range of 300-600 ml/min.

20 The pressure generator may produce mechanical or hydrostatic pressure on the tissue and may be, for example, a compressor (piston) present in the chamber, which can periodically apply pressure on the tissue present in the chamber when streaming in one direction and the pressure is released when streaming in the other direction. The compressor should be under control of a control mechanism capable
25 of controlling the timing (frequency, pausal, etc.) and the level of compression, such as a clock or a computer mechanism.

The control mechanism would trigger the compressor, to compress the chamber thus applying rhythmic pressure on the liquid present therein, and consequently applying pressure on the tissue. In the case of a compressor, the
30 pump's activity may be constant so that the medium circulates between the

chamber and the reservoir at a constant rate in order to improve gas exchange and nutrient availability to the tissue.

By another alternative, the pump that circulates the medium between the chamber and the reservoir is itself the pressure generator capable of producing rhythmic pulses of hydrostatic pressure on the tissue. In that case the pressure generator is the pump itself and no additional elements (such as a compressor) are required to produce the rhythmic pulses of pressure. The pump which is a peristaltic may have a built-in means for triggering rhythmic pulses. Alternatively, the pump may be connected to a control mechanism which triggers the duration, delays and frequencies of the pump such as a clock or a computer mechanism.

By alternating activities of such a pump, the medium can circulate in pulses between the medium reservoir and the chamber, thus creating rhythmic pressure pulses on the tissue. Preferably the direction of the medium flow should be changed (for example clock-wise and then counter-clock-wise) as changing the flow direction simulates best the joint's conditions of loading and unloading. Typically change of direction should be every 1 to 3 min.

The rhythmic pulses should have a frequency of 5-300 per min., preferably 10-200 per min., most preferably 60 to 120 per min.

The hydrostatic pressure should be between 0.5 and 30 atm., preferably 1 to 10 atm, most preferably 2 to 3 atm.

The present invention concerns a method for maintaining viable tissues, cells or explants from three-dimensional tissue, comprising placing these tissues in the chamber of the above system with any of the above parameters of pressure, frequency and change of flow direction. Examples of tissue are as defined above in connection with the scaffold.

The present invention further provides a method for maintaining viable cells or tissue explants from three-dimensional tissue comprising growing a prosthesis composed of the scaffold of the invention impregnated with cells in the system of the invention with conditions specified above (i.e. the parameters specified above). A preferable example is a method for growing fresh cartilaginous tissues such as

embryonal epiphyseal tissue, turning to an allogeneic implant upon *in vivo* transplantation.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Fig. 1 shows a schematic representation of the system of the invention for maintaining viable three-dimensional tissue;

 Fig. 2(a) shows a histological section from whole embryonal epiphyses maintained under static growth conditions in a growth medium;

 Fig. 2(b) shows histological sections from whole embryonal epiphyses
10 maintained in the perfusion system of the invention under conditions of rhythmic pressure;

 Fig. 3(a) – (d) shows histological cross-sections of scaffolds of the invention composed of demineralized cortical and spongy bovine bone modified by cross-linking, which is impregnated with human chondrocytes;

15 Fig. 4(a) – (b) shows histological cross-sections of scaffolds of the invention composed of pig small intestine submucosa (SIS) modified by cross-linking, impregnated with human chondrocytes;

 Fig. 5 shows embryonal epiphyseal tissue resulting from spontaneous fusion; and

20 Fig. 6 shows a histological section of the tissue of Fig. 5.

DETAILED DESCRIPTION OF THE PERFUSION DEVICE OF THE INVENTION

 Reference is now made to Fig. 1 which shows an embodiment of the system
25 of the invention 1. The system is composed generally of a chamber 2 which holds within scaffold 3 of the invention impregnated with exogenous cells (e.g. autologous chondrocytes or even intact embryonal epiphyses serving as future allogeneic cell implant), or for example scaffold made of embryonal epiphyseal tissue (devoided of the endogenous cells, thus impregnated with exogenous ones).
30 The system further comprises a medium-containing reservoir 4 which is filled with

a media capable of supporting and maintaining viable cells. Medium flows from chamber 4 through out flow tube 9 into pump 6 and then through tube 16 into chamber 2. The medium then returns to the reservoir through connecting chamber outflow tube 10. The circulation of the medium is mediated through pump 6. In the present example, the pump is the element creating rhythmic pressure by its rhythmic activities and is a peristaltic pump. By other examples the pump may work continuously and rhythmic pressure may be created by other independent means such as a compressor.

The chamber and the reservoir are enclosed in CO₂ equilibrium incubator 13, which maintains constant gas contact and temperature. In reservoir 4, there is a small aquarium pump 5 and filter 14 for circulating the medium in the reservoir, filtering out particles and contaminants. The reservoir also includes needle valve 8 to equilibrate and release pressure, as well as gases outlet 11. Chamber 2 includes a mechanical plunger 12.

The system also comprises control mechanism 15 which can be a computer and in this case is an electrical electronic controlling device. The computer controls the timing, duration, pausing of the activity of pump 6 as well as the direction of the flow in the system. Thus, by giving the computer correct parameters, it is possible to activate pump 6 rhythmically so that it can work and pause alternatively resulting in streaming which will cause hydrostatic pressure on the cells present in scaffold 3 in chamber 2. Furthermore, by this control the pump may change the direction of the flow of medium. It may initially flow in the direction of 4 → 9-6 → 16-2 → 10 → 4, and then the direction may be reversed so that it flows in the reverse direction.

The volume of reservoir chamber 4 is preferably about 50 times that of chamber 2.

The chamber 2 and the medium reservoir 4 are enclosed in an incubator 13 at 37°C and a pH of 7.25 ± 0.05.

Example 1 Tissue maintenance in the system of the invention

Maintenance of whole embryonal epiphyses both as a separated organ, or as several epiphyses fused together occurred in the system of the invention.

Experiments were conducted both *in vitro* and *in vivo* in an avian model, while human, embryonal epiphyses of aborted fetuses were experimental *in vitro* only. Whole epiphyses used further as implants transplanted by squeezing them (press fit) into articular defects (animal experimentation).

Maintenance was achieved by keeping the epiphyses in the device shown in Fig. 1 for 10-20 days. Vitality was assessed by histology, ³⁵S-sulfate incorporation into isolated glycosaminoglycans and by XTT test (as explained below in Example 6). The results are shown in Fig. 2(a) and 2(b).

Fig. 2(a) shows tissue prepared as above kept in an incubator under static conditions (i.e. with no effects of rhythmic pressure). As can be seen the tissue is necrotic as evident by lysis of cells and spilling out of the nuclei to the medium. Against this Fig. 2(b) shows the same tissue kept in the perfusion system of the invention. The tissue remained viable with intact cells. These results show that the system of the invention is advantageous for maintaining viable tissue for prolonged periods of time.

20 Example 2 Inducing repair in articular defects.

Defects were created in vital articular cartilage explants which were placed in a system as described schematically in Fig. 1. The explants were grown and maintained in the system for 7-14 days to induce regeneration and repair in the tissue either by transplanting cells in adhesives or whole epiphyses. The results (not shown) indicate that cell and tissue integration and propagation within the defects took place signifying that the system of the invention enables repairment of defects in tissue and development of normal new tissue.

Example 3 Scaffold preparations from embryonal epiphyses, soft and calcified connective tissue shunks

Epiphyses from aborted human fetuses (15-25 wks) were collected,
5 cleaned from soft tissues and underwent 5 cycles of freezing and thawing in
distilled water and washed thoroughly by phosphate buffered saline after each
cycle, in order to get rid of cells and cytosolic residues. These tissue residues
containing basically matrices enriched by growth factors, were further bored to
increase porosity, making holes and channels by a specially designed metal
10 brush.

Cortical and cancellous bone, articular, meniscal and tracheal cartilages all
underwent basically the same chemical-enzymatic and mechanical procedure for
their adjustment as a scaffold. The scaffolds were used for cell and tissue explant
adherence and growth, in the process of *in vitro* reconstruction of composite
15 engineered cartilaginous implants to induce regeneration and repair in damaged
articular cartilages.

1. The tissue samples were cleaned of soft tissue residues and
underwent three cycles of freezing and thawing using liquid
nitrogen for freezing and double distilled water for thawing,
20 followed by three washes in phosphate buffered saline (PBS) at
pH 7.4, getting rid of the cytoplasmic content of the lysing cells.
2. The tissue samples were further extracted by constant stirring in
4M guanidinium chloride for 48 hours in the cold room. After
thoroughly washes in dH₂O, removal of all chlorides was assessed
25 using AgNO₃.
3. Calcified tissue further underwent a decalcification step by either
EDTA, acids or special reagents, followed by thoroughly washes
with distilled water and PBS.
4. For removing lipids the samples were extracted by constant stirring
30 with chloroform:methanol (1:1 v/v) in the cold room, until no more
yellowish substances were extractable. To remove the organic

solvents the samples were washed with PBS for overnight under constant stirring in the cold.

5. A short collagenase digestion (37.5 units/ml) was performed for one hour in PBS at 37°C. The digestion was terminated and followed by rinsing twice in saline (0.9% NaCl).
 6. The collagenase treatment was followed by a short papain further (proteolytic) digestion (25µl Sigma concentrate/ml papain buffer (pH 5.4) containing cysteine (1mg/ml)).
 7. The samples were rinsed 5 times in saline enriched by antibiotics.
 8. Production of pores was performed by hammering the tissue over a scraper and rinsing with PBS.
 9. The tissue samples were kept sterile and frozen until it was collected for *in vitro* reconstruction with cells and tissue explants, designed for implantation.
- Variation of this procedure is dependent on the exact consistency of the original tissue used.

Example 4 Sugar cross-linking of matrix agents added to natural tissue to be used as scaffold

- One percent solutions of hyaluronic acid, cartilage proteoglycan – aggrecan, various glycosaminoglycans, (chondroitin sulfates, keratan sulfates, heparan sulfates (syndican and perican), dextran sulfate (synthetic), egg lysozyme, polylysine (synthetic), arteparon ect., were mixed with either 1% ribose or 1% xylose in the cold in suspension with the treated tissue scaffold, for 3 days in the cold room. Then the tissue samples were washed thoroughly in PBS.

Example 5 Implant scaffolds impregnated with cells

Chondrocytes from human arthroscopic biopsies, perichondrium and periosteum were cultured to a cell density of 10^7 cells/cm². Scaffold enriched with matrix constituents were formed from one of the following: (1) demineralized bone matrix; (2) modified cross-linked small intestine submucosa and (3) acellular matrices of embryonal epiphyses.

Demineralized cortical and spongy bone was treated mechanically to produce porosity by hammering the tissue over a scrapper device.

Pig small intestine submucosa (SIS) deficient of its endothelial layer was modified by guanidinium chloride extraction, and sugar cross-linking as explained above. Hyaluronic acid and chemically altered hyaluronic acid molecules. The above human arthroscopic derived differentiated cultures were used as cell source for impregnating the scaffold.

Human embryonal epiphyses were treated to eliminate (devitalize) the cells, thus leaving the matrices and their absorbed molecules (e.g. growth factors) intact. Increased porosity was obtained by booring a metal brush into the tissue.

The results are shown in Fig. 3(a)-3(b) which show several different histological preparation of demineralized cortical and spongy bovine bones impregnated in cells and in Fig. 4(a) and 4(b) which show a pig small intestine submucosa (SIS) devoid of its endothelial layers modified by guanidinium chloride extraction and cross-linked as above.

As can be seen, the two types of the above scaffolds of the invention supported maintenance of viable cells, show normal morphology.

Example 6 Embryonal epiphyses as implants to repair articular cartilage defects with and without embedding in a scaffold.

Fresh embryonal epiphyses were isolated and cleaned of soft tissues of long bones tibias and femurs of aborted fetuses at medium gestation and up to birth (the younger gestation age the better). The isolated and cleaned epiphyses can be

maintained vital for weeks, up to the need for implantation, under a constant perfusion in the system of the invention. The separated epiphyses tend under these cultured conditions to associate (fuse together) into one tissue piece. The vitality of the newly formed tissue compared to epiphyses maintained under

5 regular static culture conditions was assessed by:

- (a) Histology, histochemistry and immunohistochemical staining procedures,
- (b) Incorporation of ^{35}S -carrier free sulfate into isolated glycosaminoglycan macromolecules, and
- 10 (c) By XTT reagent measuring cell vitality in a standard tissue ring specimens by reduction of tetrazolium salts by the cell's mitochondrial hydrogenases, yielding a soluble colored molecule – a formazan dye, measured at 450 nm with an ELISA plate reader. The optical density is proportional to the number of living cells and their
- 15 metabolic status.

These biological in vitro reconstructed (engineered) tissues have clear advantages as implants as follows:

- (a) The engineered in vitro reconstructed cartilaginous implants are readily malleable to fit the exact shape and size of the articular
- 20 defects.
- (b) They are readily squeezed in (press fitted) and strongly held (anchored) into the lesion sites, integrating rapidly with the neighboring cartilage and bone tissues.

Fig. 5 shows epiphyseal tissue which has been spontaneously fused, maintained and in the system of the invention demonstrates that the system of the invention can maintain relatively large pieces of tissue in viable state for extended periods of time. Fig. 6 shows a histological cross-section of the tissue of Fig. 5. As can be seen, the system of the invention supports the viability of a large range of different cells at a wide range of differentiated stages as evident

from the fact that various stages of differentiation were maintained in a viable state.

CLAIMS:

1. A scaffold for use as growth supportive base for cells and tissue explants form a three-dimensional tissue, comprising: naturally occurring connective or skeletal tissues derived from biological tissues which have been treated for
5 elimination of cellular or cytosolic elements and which have been modified by cross-linking with at least one agent selected from the group consisting of: hyaluronic acid, proteoglycans, glycosaminolycans, chondroitin sulfates, heparan sulfates, heparins and dextran sulfates.
2. A scaffold according to Claim 1, wherein the cellular or cytosolic elements
10 are: DNA, RNA, peptides, lipids and proteoglycans.
3. A scaffold according to Claim 1, wherein the connective or skeletal tissue has been treated to increase its porosity.
4. A scaffold according to Claim 3, wherein the connective or skeletal tissue has been treated to increase its porosity by mechanical drilling or mechanical
15 pinching of pores.
5. A scaffold according to Claim 3, wherein the treatment for increasing porosity comprises incubation with chemical or biological agents capable of controlled partial degradation of the biological tissue.
6. A scaffold according to Claim 1, wherein said cross-linking is carried out by
20 an agent selected from the group consisting of: sugar base substances, carbodimide acterone, or 1,1,carbonyl dimidazole.
7. A scaffold according to Claim 6, wherein the sugar based substances is ribose or xylose.
8. A scaffold according to Claim 1, where the naturally connective or skeletal
25 tissue is selected from the group consisting of: demineralized bone and subinternal layer of intestine, epiphysis, periochondrium and periosteum
9. A scaffold according to Claim 1, further comprising at least one compound selected from the group consisting of: adhesive molecules and growth factors.
10. A scaffold according to Claim 9, wherein the adhesive molecules are
30 selected from the group consisting of: integrines, laminin, fibronectin, hyaluronic acid, polylysine and lysozyme.

11. A scaffold according to Claim 9, wherein the growth factors are selected from the group consisting of: FGF's, TGF's, BMP's, and IGF's.
12. A scaffold according to any one of the preceding claims, impregnated with cells forming a three-dimensional tissue.
- 5 13. A scaffold according to Claim 12, wherein the three-dimensional tissue is composed of cells selected from the group consisting of: mesenchymal tissue, articular cartilage, epiphyseal tissue, perichondrial and periosteal flaps, skeletal tissue, liver tissue, neuronal tissue, dermis tissue and fibrous tissue.
14. A prosthesis for implanting into a body of an individual comprising as an
10 active ingredient the scaffold of Claim 12.
15. A prosthesis for implanting into a body of an individual comprising as an active ingredient the scaffold of Claim 13.
16. A system for the maintenance of viable tissue comprising:
 - 15 (i) a chamber for holding the tissue, the chamber's atmosphere being kept at a relatively constant gas composition, said gas composition being suitable for maintenance of viable biological tissue;
 - (ii) a reservoir for holding tissue culture medium, said reservoir being in flow communication with the chamber;
 - (iii) a pump for circulating the medium between the chamber and the
20 reservoir in a controlled manner; and
 - (iv) a pressure generator for producing rhythmic pulses of pressure on the tissue present in the chamber.
17. A system according to Claim 16, wherein the tissue is a three-dimensional tissue.
- 25 18. A system according to Claim 16, further comprising control mechanism for controlling the duration and frequency and pausing of the pressure generator.
19. A system according to Claim 16, wherein the pressure generator is a compressor.
20. A system according to Claim 16, wherein the pressure generator is a pump
30 of (iii) which works rhythmically.

21. A system according to Claim 16, wherein the direction of flow of the medium between the chamber and the reservoir is constantly altered.
22. A system according to Claim 16, wherein the frequency of the rhythmic pressure pulses is 5-300 per min.
- 5 23. A system according to Claim 22, wherein the frequency is 10-200 per min.
24. A system according to Claim 23, wherein the frequency is 60-120 per min.
25. A system according to Claim 16, wherein the pressure is 0.5 to 30 atm.
26. A system according to Claim 25, wherein the pressure is 1 to 10 atm.
27. A system according to Claim 26, wherein the pressure is 2 to 3 atm.
- 10 28. A method for maintaining viable a three-dimensional tissue explant, viable organs, or viable cells from three-dimensional tissue comprising: placing the tissue, organs or cells in the chamber of the system of Claim 16 and activating the pressure generator to produce rhythmic pressure pulses on the tissue, organ or cells.
29. A method for maintaining viable three-dimensional tissue comprising
- 15 placing the prosthesis of Claim 14 in the chamber of the system of Claim 16 and activating the pressure generator to produce rhythmic pressure on the prosthesis.
30. A method according to Claim 29, wherein the prosthesis comprises a scaffold impregnated with epiphyseal tissue.
31. A method according to Claim 28, wherein the viable three-dimensional
- 20 tissue is epiphyseal tissue or embryonic derived from aborted fetuses and adult.
32. A method according to Claim 28, wherein the frequency of the rhythmic pressure pulses is 5-300 per min.
33. A method according to Claim 32, wherein the frequency is 10-200 per min.
34. A method according to Claim 33, wherein the frequency is 60-120 per min.
- 25 35. A method according to Claim 28, wherein the pressure is 0.5 to 30 atm.
36. A method according to Claim 35, wherein the pressure is 1 to 10 atm.
37. A method according to Claim 36, wherein the pressure is 2 to 3 atm.

1/7

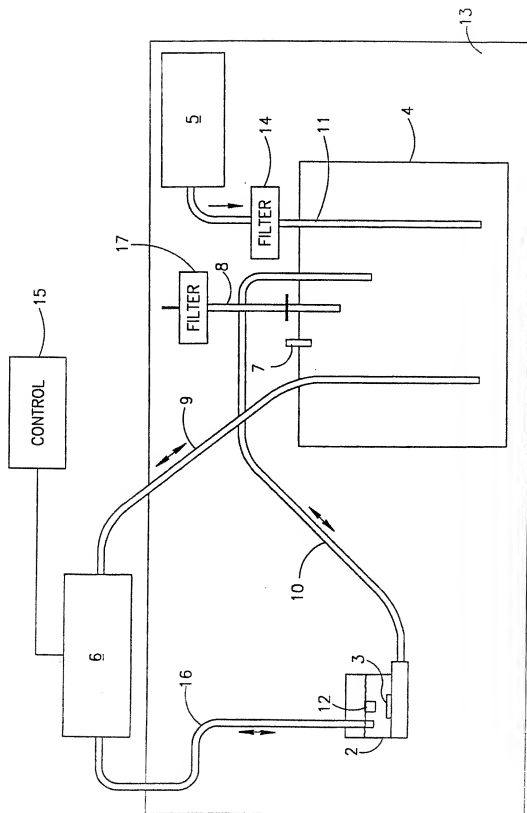


FIG.1

2/7

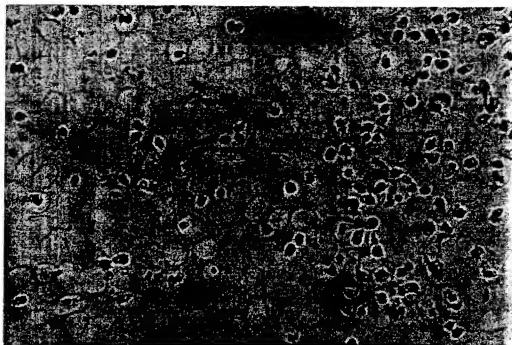


FIG. 2A

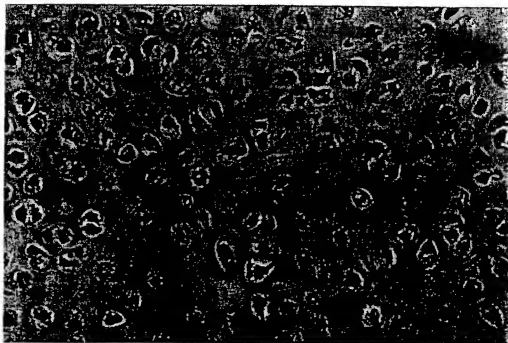


FIG. 2B

3/7



FIG. 3A



FIG. 3B

4/7

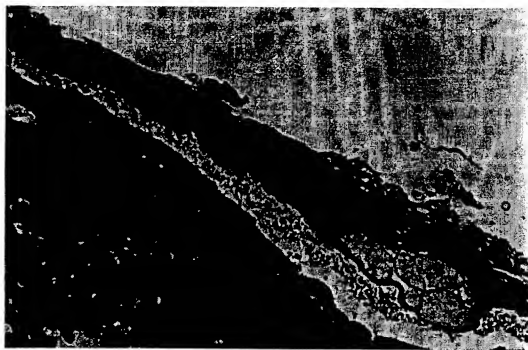


FIG. 4A

5/7



FIG. 4B

6/7



FIG. 5

7/7

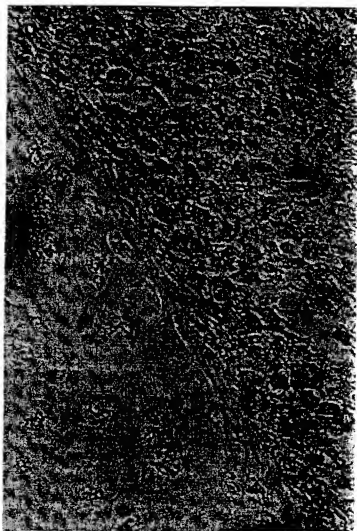


FIG. 6